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Effect of Molecules Secreted by *Lactobacillus acidophilus* Strain La-5 on *Escherichia coli* O157:H7 Colonization▽

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The probiotic bacterium *Lactobacillus acidophilus* strain La-5 is a gut-colonizing microorganism that, when established, becomes an important part of the gastrointestinal (GI) tract microbiota. It has been shown to be effective against enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 infection. We have previously shown that molecules released by probiotic strain La-5 influence the transcription of EHEC genes involved in colonization and quorum sensing. In this work, we report on the ability of these molecules to prevent the adherence of EHEC to epithelial cells and on its capacity to concentrate F-actin at adhesion sites. With a fluorescein-labeled phallotoxin, it was shown that La-5 cell-free spent medium (CFSM) fractions remarkably reduced attaching and effacing lesions in HeLa cells. We also observed a significant inhibition of bacterial adhesion to Hep-2 cells when they were treated with the same La-5 CFSM fractions. In order to observe if La-5 CFSM fractions exhibited the same effect in vivo, we studied the ability of luminescent EHEC constructs (*LEE1*::*luxCDABE*) to adhere to intestinal epithelial cells of specific-pathogen-free ICR mice following intragastric inoculation. Colonization of the GI tract by luminescent EHEC O157:H7 was monitored in real time with a slow-scan charge-coupled device camera. At the same time, fecal shedding of EHEC was studied. Following oral gavage of the La-5 active fraction, we observed a reduced amount of bioluminescence signal along with a decrease in fecal shedding by mice, indicating an effect on the ability of the organism to colonize the GI tract. Our results confirm past evidence of the possibility of blocking or interfering with EHEC's virulence by active molecules contained in the probiotic CFSM and identify novel therapeutic alternatives to antibiotic treatments in the fight against food-borne pathogens.

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC O157) is a member of the attaching and effacing (AE) *E. coli* group (3). These strains form specific structures known as AE lesions in the host intestinal epithelial wall which allow EHEC O157 to intimately attach to the epithelial membrane in order to achieve colonization (18, 22, 24). The complex model of AE lesion formation has been extensively studied (8). Initial attachment of the bacterium is followed by the injection of bacterial proteins into the host cell (17, 21) through a specialized translocation apparatus termed a type III secretion system. This results in the cytoskeletal rearrangement and effacement of the microvilli. Finally, a 94-kDa bacterial outer membrane protein termed intimin is required (19) that results in the formation of the bacterium-host cell pedestal structure (9, 10, 27, 37). A number of enteric bacteria, including EHEC, are known to produce and/or respond to chemical signals called autoinducers. The use of this cell-to-cell signaling mechanism facilitates the regulation of important traits of enteric microbes that allow them to successfully colonize and/or start infection in their host (20). EHEC virulence-specific genes are regulated by quorum sensing (QS) (34, 35) mediated by the autoinducer 3 (AI-3)/epinephrine/norepinephrine signaling system (36). AI-3 is a molecule produced by the commensal gastrointestinal (GI) microbiota that seems to resemble the hormones epinephrine and norepinephrine produced by the host (36). This resemblance is believed to allow enteric pathogens to organize a concerted activation/repression of specifically required genes. Furthermore, the discovery of an EHEC sensor kinase, QseC, which binds AI-3 and the hormones epinephrine and norepinephrine and regulates virulence in a rabbit infection model provides evidence that this QS system participates in interkingdom cross-communication (5). Thus, enteric pathogens possess an extremely complex regulatory system that is used to systematically compete in such a challenging environment and inhibition of this QS system may lead to an attenuation of virulence.

Probiotic bacteria have been shown to prevent EHEC infection in mouse models (2, 11, 31), but the molecular basis of their mode of action is still not entirely understood. We propose that probiotics produce small biologically active molecules that are able to interfere with cell-to-cell signaling between bacteria occupying their niche. The capacity of probiotics to inhibit the attachment of certain pathogenic bacteria to intestinal epithelium and their quorum quenching strategies against EHEC could offer potential novel therapeutic approaches to combat this pathogen. Here we report the effect of chromatographically selected fractions of *Lactobacillus acidophilus* La-5 on the ability of EHEC O157 to adhere to and cause AE lesions on mammalian cells.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are described in Table 1. *L. acidophilus* strain La-5 was grown under anaerobic conditions at 37°C

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TABLE 1. Bacterial strains and the construct used in this study

Strain or construct	Serotype	Relevant genotype or property	Reference
E. coli strains			
VS94	O157:H7	*luxS* mutant	21
43894	O157:H7	$Stx1^+$ $Stx2^+$; isolated from human stool in Michigan	CRIFS stock[a]
L. acidophilus La-5		Probiotic lactic acid bacterium	CRIFS stock
E. coli C4 construct	O157:H7	Derived from ATCC 43894; $Stx1^+$ $Stx2^+$ LEE2::*lux*	15

[a] CRIFS stock strains are deposited in the Canadian Research Institute for Food Safety culture collection.

in modified DeMann, Rogosa, and Sharpe medium (29). *E. coli* O157:H7 strains VS94 (36) and 43894 were grown in Luria-Bertani (LB) broth (BD Diagnostic Systems), and bioluminescent strain *E. coli* O157 C4 was grown in LB agar supplemented with ampicillin (AMP) and kanamycin (KM) (Sigma-Aldrich Canada Ltd.), each at a concentration of 50 μg/ml, and both strains were incubated overnight at 37°C. A single colony of each *E. coli* O157 strain was taken from the plate and subcultured in either LB broth or high-glucose Dulbecco's minimum essential medium (Sigma-Aldrich Canada Ltd.), and only the bioluminescent *E. coli* O157 C4 strain was supplemented with the antibiotics. The strains were then incubated overnight at 37°C on a shaker at 150 rpm. The correlation between luminescence and cell count in LB broth was established by a standard plate count technique and by the measurement of bioluminescence in 1 ml of culture serial dilutions with a tube luminometer (MGM Instruments, Hamden, CT). For the infection of mice, an overnight culture of the *E. coli* O157 C4 strain was centrifuged at 13,000 × *g* for 10 min, washed, and resuspended in fresh, antibiotic-supplemented LB broth.

***L. acidophilus* La-5 cell-free fractions.** Cell-free fractions were prepared as previously described (29). Briefly, *L. acidophilus* strain La-5 was grown overnight in modified DeMann, Rogosa, and Sharpe medium [10 g peptone from casein, 8 g meat extract, 4 g yeast extract, 8 g D(+)-glucose, 2 g dipotassium hydrogen phosphate, 2 g diammonium hydrogen citrate, 5 g sodium acetate, 0.2 g magnesium sulfate, and 0.04 g manganese sulfate in 1 liter distilled water; BD Diagnostic Systems, Sparks, MD]. The overnight culture was diluted 1:100 in fresh medium. When the culture grew to an optical density at 600 nm of 1.6 (1.2×10^8 cells/ml), the cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. The supernatant was sterilized by filtering through a 0.2-μm-pore-size filter (Millipore, Bioscience Division, Mississauga, Ontario, Canada) and will be referred to as cell-free spent medium (CFSM). Two liters of *L. acidophilus* La-5 CFSM was collected and freeze-dried (Unitop 600 SL; VirTis Co., Inc., Gardiner, NY). The freeze-dried CFSM was reconstituted with 200 ml of 18-Ω water. Freeze-dried CFSM was stored at −20°C prior to the assays. The total protein content of the reconstituted CFSM was quantified with Bio-Rad *DC* protein assay kit II (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada).

Fractionation of *L. acidophilus* La-5 CFSM. Five milliliters of CFSM was directly deposited onto a P2 Biogel (Bio-Rad, Mississauga, Ontario, Canada) column (exclusion, 100 to 1,800 Da; 2.5 by 100 cm; Bio-Rad Laboratories Ltd.) and run at room temperature in 18-Ω water at a gravity flow rate of 0.8 ml/min, and 80 5-ml fractions were collected. The fractions collected were freeze-dried and resuspended in 1 ml 18-Ω water for preliminary screening against EHEC production of AI-2 and expression of operons 1 and 2 of the locus of enterocyte effacement (LEE) pathogenicity island (LEE1 and LEE2, respectively) as previously described (29). Fractions showing strong inhibitory activity against LEE expression and AI-2 production were selected for further analysis (data not shown). The total protein content of the pooled CFSM active fractions was quantified with Bio-Rad *DC* protein assay kit II (Bio-Rad Laboratories Ltd.).

Effects of pH, heat, and enzymes on La-5 CFSM active fractions. To determine the effects of pH, heat, and proteolytic enzymes on La-5 CFSM activity against EHEC O157 C4, the active fractions were exposed to the following treatments. The CFSM pH was adjusted to 2.0 to 10.0 with 5 N NaOH or HCl, and the active fractions were heated at 100°C for 5, 10, or 20 min and at 121°C for 15 min and treated with the following enzymes at a final concentration of 1 mg ml^{-1}: trypsin (pH 7.0; Sigma-Aldrich, Canada), pepsin (pH 3.0; Sigma-Aldrich), proteinase K (pH 7.0; Sigma-Aldrich), and lysozyme (pH 7.0; Sigma-Aldrich). After incubation at 37°C for 2 h, enzyme activity was inactivated by heating at 100°C for 5 min. Untreated samples were used as controls. Fractions were assayed for activity against EHEC O157 production of AI-2 and expression of LEE1 and LEE2 as previously described (29).

Fluorescent staining of actin filaments. Fluorescent actin staining tests were performed as described previously (23), with some modifications. HeLa human cervix adenocarcinoma epithelial cells were provided by Roger Johnson (Laboratory for Foodborne Zoonoses, Public Health Agency of Canada). HeLa cells were grown in complete Eagle's minimal essential medium (EMEM; Sigma-Aldrich Canada Ltd.) supplemented with 2% (vol/vol) fetal bovine serum (Invitrogen Canada Inc.). Cells were then plated onto four-well microchamber slides at 2×10^5 ml^{-1} and incubated for 24 h at 37°C in the presence of 5% CO_2. The cells were then maintained during the assay in serum- and antibiotic-free EMEM. Before inoculation with bacteria, selected individual fractions of *L. acidophilus* CFSM (F33 and F34) were added to treatment group wells. As a negative control for attachment and effacement (AE) lesion formation, we used *E. coli* O157:H7 strain VS94, which is LuxS negative. The negative control group wells were inoculated with 10^5 *E. coli* O157:H7 strain VS94 bacteria with or without supplementation with 100 μM propranolol and with only the selected fractions of *L. acidophilus*. Propranolol was used to suppress complementation of the AE phenotype by the hormones epinephrine and norepinephrine produced by the eukaryotic cells. After inoculation of the bioluminescent EHEC O157 strain into treatment and positive control wells, the slides were incubated for 3 h at 37°C in the presence of 5% CO_2. The cells were then washed three times with phosphate-buffered saline (PBS), and fresh medium plus the treatments were added. Cells were incubated for another 3 h and then washed six times with PBS and fixed in 4% paraformaldehyde. Fixed and washed cells were permeabilized by treatment of slides with 0.1% Triton X-100 in PBS for 15 min. Cells were incubated with 0.2% bovine serum albumin (Invitrogen Canada Inc.) in PBS for 1 h at room temperature. After three washes in PBS, slides were treated with a 10-μg/ml solution of fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma-Aldrich Canada Ltd.) in PBS for 40 min at room temperature to specifically stain actin filaments. Slides were washed three times in PBS and then examined with a Zeiss Axioskop 2 with fluorescence filters for FITC. Images were recorded with the Axiocam and Zeiss Axiovision Software.

HEp-2 cell adhesion assay. In order to compare levels of adherence to HEp-2 epithelial cells in culture, we used an established model for evaluating adherence of EHEC O157 (27). HEp-2 human laryngeal carcinoma epithelial cells were a kind gift from Carlton Gyles (Department of Pathobiology, University of Guelph). Briefly, HEp-2 cells grown in EMEM supplemented with 10% (vol/vol) fetal bovine serum were plated onto 24-well tissue culture plates at 2×10^5 ml^{-1} and incubated for 24 h at 37°C in the presence of 5% CO_2. The cells were then maintained during the assay in serum- and antibiotic-free EMEM. Before inoculation with bacteria, a 10% (vol/vol) concentration of the *L. acidophilus* CFSM pooled active fractions (F33 and F34) was added in triplicate to treatment group wells. Wells containing the negative control groups were inoculated with 10^5 *E. coli* O157:H7 strain VS94 with or without supplementation with 100 μM propranolol (Sigma-Aldrich Canada Ltd.). Following the inoculation of 10^5 EHEC O157 cells into treatment and control group wells, the plates were incubated for 3 h at 37°C in the presence of 5% CO_2. The cell monolayers were then washed three times with PBS to remove nonadhering bacteria, and fresh medium and the treatments were added. Cells were incubated for another 3 h and then washed six times with PBS. Washed cells were lysed with 0.1% Triton X-100. Released bacteria present in the suspension were collected, and appropriate dilutions were plated onto LB agar. To evaluate if the percentage of adherence in the treatment groups was significantly different from that in the control group, where the counts recovered from the control group (2.2×10^7 CFU ml^{-1}) were considered to be 100%, the percentage of adherence in the negative control and treatment groups was calculated with the following equation: % Recovery = (Group CFU ml^{-1} × 100)/2.2×10^7.

Mouse colonization experiments. Specific-pathogen-free female ICR mice were obtained at 3 weeks of age from Taconic Farms (New Jersey) and used for the experiments after 1 week of acclimation. Mice were housed at the isolation unit of the Central Animal Facility (University of Guelph) in a temperature-controlled environment with a 12-h light-dark cycle. Animal care was provided in accordance with animal utilization protocol 04R030 (University of Guelph) and the *Guide to the Care and Use of Experimental Animals* (1). Mice were fed sterilized solid rodent chow and water. When needed, the water was supplemented with AMP and KM at concentrations of 400 and 200 mg/liter, respectively. Each mouse was assessed daily for weight, body temperature, signs of dehydration, posture, and alertness.

Dose-response experiments. Ten mice were divided into five groups of two, and each group was infected by oral gavage with 100 μl of a bacterial cell suspension containing 10^5 to 10^9 cells. Mice were given the antibiotics required for selection of the *luxCDABE*-encoding plasmid in their drinking water at the concentrations mentioned previously. Sucrose (5%, wt/vol; Sigma-Aldrich Canada Ltd.) was added in order to make the water supplemented with the antibiotics palatable. The 5% sucrose solution supplemented with the antibiotics was changed daily.

Feeding-infection experiments. Mice were divided into four groups. Group 1 was fed 100 μl of La-5 pooled active fractions (F33 and F34) (negative control; $n = 5$); groups 2 and 3 were fed daily with 100 μl of La-5 pooled active fractions (F33 and F34) 2 days before (probiotic-EHEC) and 2 days after (EHEC-probiotic) a challenge with EHEC O157 C4, respectively ($n = 5$); and group 4 (positive control) was infected with 10^8 CFU ml^{-1} EHEC O157 C4 ($n = 5$). Feeding-infection experiments were repeated three times. Groups 2 to 4 were infected by oral gavage with 100 μl of the 10^8 CFU ml^{-1} bacterial cell suspension on day 3 of the experiment. All of the groups were kept under climatization conditions, except the mice in group 2, which 2 days prior the bacterial challenge were fed 100 μl of the La-5 active fractions.

Bioluminescent imaging. Bioluminescent imaging was performed as previously described (4), with minor modifications. Briefly, bioluminescent imaging was monitored on days 3, 5, and 7 after infection. Prior to imaging, mice were anesthetized with a cocktail composed of ketamine (60 mg/kg) and medetomidine (0.75 mg/kg). Atipamezole (2.25 mg/kg) was used to reverse the effects of the anesthetics. All drugs were administered intraperitoneally. Both bioluminescent and photographic images of mice were taken with a cooled slow-scan charge-coupled device camera (NightOWL Molecular Imager; EG&G Berthold Technologies, Wildbad, Germany). The integration time for bioluminescence was 1 min at low resolution. Images were processed with the WinLight software (EG&G Berthold). Pseudocolor images were obtained to represent the distribution of bioluminescent intensity, which changed from blue to yellow to red with increasing light output. Bioluminescent images were superimposed onto photographic images of the same mice to locate the origin of the bioluminescence. The areas of maximum bioluminescence were identified with the use of the two-dimensional peak search option of the software, and light output from these areas was calculated in terms of relative light unit counts per square centimeter per second [cts $(cm^2\ s^{-1})^{-1}$] with the WinLight program. The dose-response experiment was carried out over 7 days. The feeding-infection experiment was carried out over 12 days or until the end point of the experiment (indicated by a body temperature of <34°C and/or loss of 20% of body weight) had been reached. At the end point, mice were euthanized with CO_2.

Enumeration of EHEC O157 bacteria shed in feces. Fresh feces of mice were weighed and suspended in PBS (0.5 g of feces per 4.5 ml of 0.1% [wt/vol] sterile peptone water) to obtain a concentration of 100 mg ml^{-1}. The fecal suspensions were serially diluted 10-fold, and appropriate dilutions were plated in triplicate onto LB agar supplemented with 50 μg ml^{-1} AMP and KM. Colonies that developed after incubation for 24 h at 37°C were counted. The limit of detection was 10^2 CFU g of $feces^{-1}$. A value of 10^2 g of $feces^{-1}$ was assigned to any culture showing no detectable colonies for the purpose of deriving statistical data.

Statistical analysis. All of the results in this report are means of three independent trials ± standard deviations. The Student *t* test was used when necessary to assess the statistical significance of the differences between test and control groups ($P < 0.05$).

RESULTS AND DISCUSSION

Fractionation of *L. acidophilus* La-5 CFSM. Fractions 33 to 35 of *L. acidophilus* La-5 CFSM showed strong inhibitory activity against LEE1 and LEE2 expression and AI-2 production (data not shown). These fractions were selected for further studies. The total protein content of the CFSM before fractionation was 9.7 mg/ml, and after fractionation, the total protein content of the pooled active fractions was 4.1 mg/ml.

TABLE 2. Factors affecting the inhibitory activity of *L. acidophilus* CFSM toward EHEC O157:H7 LEE expression and AI-2 production

Treatment	Activity[a] of CFSM against EHEC O157:H7	
	LEE1/LEE2 expression	AI-2 production
Proteinase K (0.1 mg ml^{-1})	33	37
Pepsin (0.1 mg ml^{-1})	31	34
Trypsin (0.1 mg ml^{-1})	95	95
pH 2.0–10.0	95	95
Heat	95	95
Nontreated CFSM	100	100

[a] Activity is reported as a percentage.

Enzymatic, temperature, and pH treatments of probiotic CFSM. Partial inactivation of inhibitory activity against EHEC O157 LEE expression and AI-2 signaling molecule production was observed after the treatment of biologically active CFSM fractions with proteinase K and pepsin (Table 2). No reduction of activity was found after treatment with trypsin (Table 2). There was no significant decrease in activity recorded after heat treatment (Table 2). The activity remained after 2 h of incubation at different pH values (2.0, 4.0, 6.0, 7.0 8.0, 9.0, and 10.0) (Table 2). None of the CFSM had any antimicrobial activity against EHEC O157, as inhibition of growth was not observed throughout this study.

Our results demonstrated that the *L. acidophilus*-secreted molecules were not affected by changes in culture pH and that the molecules are heat resistant. The partial inactivation of activity observed after addition of proteinase K and pepsin suggests that they might be small molecules that could consist of short amino acid chains. Nonetheless, these results do not confirm that the active molecules are proteinaceous, and there is ongoing work toward finding out the nature of these molecules.

***L. acidophilus* La-5 CFSM decreased *E. coli* O157:H7 attachment to tissue culture cells.** It was previously demonstrated that *L. acidophilus* La-5-secreted molecules influenced the EHEC O157 type III secretion system (29). We detected the downregulation of important virulence-related gene expression after EHEC O157 was grown in medium supplemented with biologically active fractions of *L. acidophilus* La-5 CFSM compared with EHEC O157 grown in the same medium without the addition of La-5 fractions. In the present study, we tested whether the addition of La-5 biologically active CFSM fractions would have an influence on EHEC O157 adhesion to eukaryotic cells in vitro and in vivo. Adhesion and AE lesion formation in eukaryotic cells (HEp-2 and HeLa cell lines, respectively) were substantially reduced when La-5 fractions were added before exposure to *E. coli* O157:H7 strain ATCC 43894. Infection of HeLa cells with EHEC O157 alone showed typical localized adherence behavior (Fig. 1A). However, when it was coincubated with La-5 fractions F33 and F34, we could visualize the reduction of actin accumulation underneath attached bacteria (Fig. 1C). HeLa cells infected with EHEC O157 $LuxS^-$ strain VS94 in the presence of propranolol showed no evidence of actin accumulation (Fig. 1D) compa-

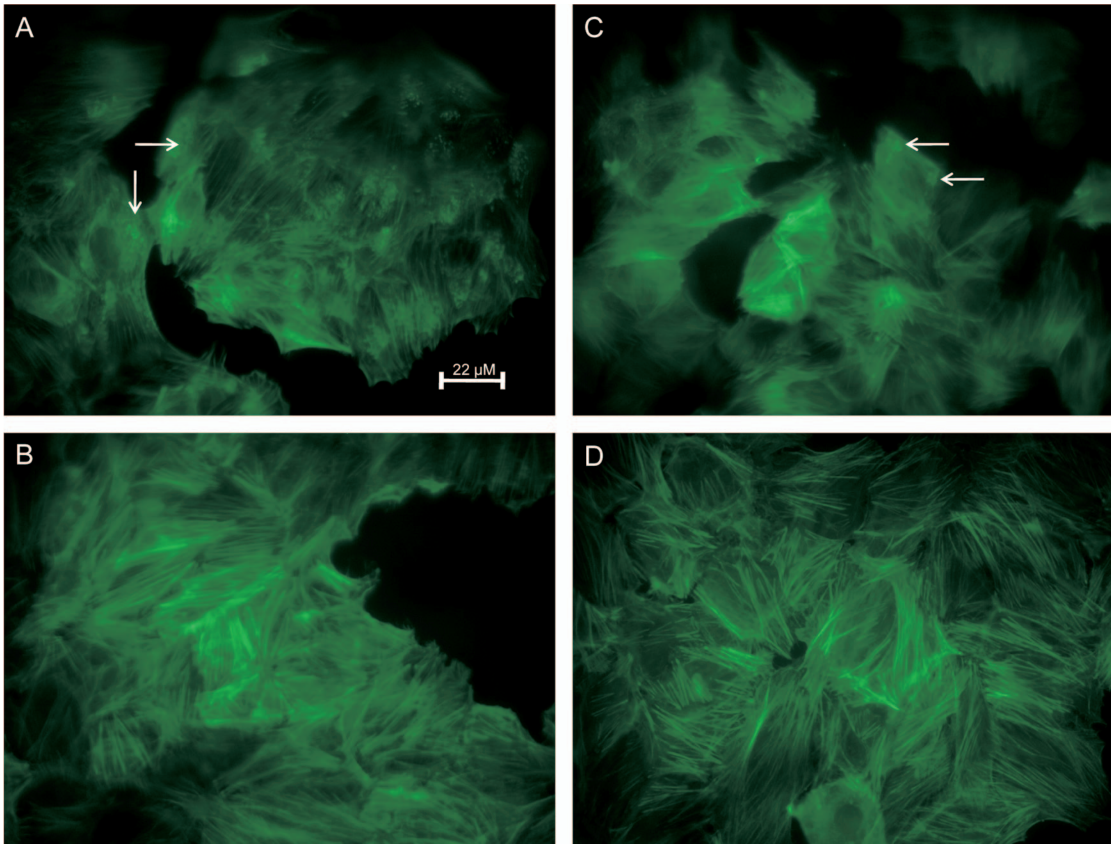

FIG. 1. Fluorescent micrographs of HeLa cells incubated for 6 h with EHEC ATCC 43984. Bright fluorescence of the FITC-phalloidin stain, indicating aggregation of foci of alpha-actinin underneath adherent EHEC 43894 microcolonies (arrows), was visualized by fluorescence microscopy. (A) Positive control (cells exposed to EHEC 43894). (B) Negative control. (C) EHEC 43894-infected cells coincubated with 40 µl of the peptide fraction of *L. acidophilus* La5. (D) AE negative control (cells exposed to LuxS$^-$ EHEC). Original magnification, ×40. Bar, 22 µm. Images are representative of three independent assays.

rable to that of noninfected HeLa cells incubated only with the La-5 CFSM selected fraction (F34) (Fig. 1B). To complement the fluorescent actin staining test, we performed an adhesion assay with the same EHEC O157 strain, 43894, and the HEp-2 cell line. The results of the adhesion assay are summarized in Table 3. Infection of HEp-2 cells with EHEC O157 was normalized to 100% for comparison with La-5-treated cells. The degree of attachment was reduced by 76% in the wells containing La-5 biologically active fractions.

TABLE 3. Adherence of EHEC O157 strain 43894 to HEp-2 cells

Bacterium	% Adherence[b]
EHEC 43894	100[a]
EHEC 43894 coincubated with 10% *L. acidophilus* La5 fraction 33	26[c]
EHEC 43894 coincubated with 10% *L. acidophilus* La5 fraction 34	24[c]
EHEC VS94 LuxS$^-$ + β blocker	22[c]
EHEC VS94 LuxS$^-$ with no β blocker	64

[a] The number of CFU per milliliter in the EHEC 43894 control group was normalized to 100% adherence ability.
[b] The results are average values of three independent replicates.
[c] Statistically significantly different ($P = 0.001$ [Student *t* test]).

Adherence of EHEC O157 to human epithelial cells involves the activation of the adhesin intimin, an outer membrane protein encoded by the *eae* gene (9, 26, 27, 37). Previous work (27) showed that production of intimin-specific antisera blocked the adherence of EHEC to HEp-2 cells. The immunogenic capacity of intimin has been intensively studied in order to develop anti-EHEC and anti-EPEC vaccines (6, 7, 12, 28). Our results showed that probiotic bacteria could be used to prevent EHEC adherence to epithelial cells in tissue culture models. The mode of action of probiotic-secreted molecules is still not clear; however, evidence shows that they may act by blocking QS mechanisms in EHEC, which results in an attenuation of its virulence (29).

Relationship between infectious dose of EHEC and bioluminescent imaging in ICR mice. A pilot study was conducted to determine the optimal infectious dose of EHEC for the bioluminescent imaging of bacterial colonization of specific-pathogen-free ICR mice. Five different cell concentrations, ranging from 10^5 to 10^9 cells per dose, were used for a single challenge with an EHEC O157 bioluminescent strain. The bioluminescent signal for mice infected with 10^5 cells was very

TABLE 4. Maximum bioluminescence levels in EHEC C4-infected mice

Mouse exptl group or dose (CFU) of EHEC (day)	Mean bioluminescence [cts (cm^2 s^{-1})$^{-1}$][a]
4, EHEC (3)	4,002 ± 544[d]
3, EHEC-probiotic (3)	5,171 ± 637[d]
2, probiotic-EHEC (3)	4,065 ± 884[d]
4, EHEC (5)	21,965 ± 4,871[c]
3, EHEC-probiotic (5)	2,176 ± 635[c]
2, probiotic-EHEC (5)	792 ± 82[c]
4, EHEC (7)	NA[b,e]
3, EHEC-probiotic (7)	875 ± 172[e]
2, probiotic-EHEC (7)	422 ± 1,493[e]
Dose-response assay	
10^5 (3)	1,900 ± 178
10^6 (3)	2,683.8 ± 65
10^7 (3)	3,364.85 ± 450
10^8 (3)	5,262.8 ± 391
10^9 (3)	27,998 ± 3,059

[a] Areas of maximum bioluminescence were calculated in terms of relative light unit counts per square centimeter per second. The results are means ± standard deviations of three replicates (n = 5).
[b] NA, not applicable.
[c] Statistically significantly different ($P < 0.05$ [Student t test]).
[d] Not statistically significantly different ($P > 0.05$ [Student t test]).
[e] Control group mice did not survive to this point.

weak throughout the experiment, and only upon inoculation with 10^7 or more CFU was the signal strong enough to be visualized and computed (Table 4). Based on previous work in which EHEC O157 proliferated in mouse intestines within 24 h of infection (2), we expected that a dose of 10^5 CFU would have been enough to elicit strong light output. Since our aim was to monitor EHEC O157 colonization in vivo in a short period of time, an inoculation dose of 10^8 CFU was selected for our challenge studies.

L. acidophilus **La-5 biologically active fraction reduces attachment of EHEC to intestinal epithelium of ICR mice.** The abilities of EHEC O157 to colonize mice treated with the probiotic La-5 active fraction and nontreated ICR mice were compared. EHEC O157 was recovered from the feces of all groups of mice infected with the organism (i.e., groups 2, 3, and 4) throughout the study. Mice in group 2 (probiotic-EHEC) showed the greatest decline in fecal shedding of EHEC C4; as of day 7 postinfection, they showed average fecal shedding of 10^4 CFU/ml compared to the 10^7 and 10^8 CFU/ml shed by group 3 (EHEC-probiotic) and 4 (EHEC) mice, respectively. We did not observed a significant decline in fecal shedding as of day 7 postinfection within groups 3 and 4, although mice in group 3 survived past day 7 and their average daily fecal shedding was comparable to that of group 2 as of the end of the study (~10^4 CFU/ml). As observed, the proportion of mice shedding EHEC O157 declined significantly over the course of the study in animals that received the La-5 fraction (groups 2 and 3; P = 0.0004 and P = 0.002, respectively); however, the fecal shedding in mice that were infected with EHEC O157 in the absence of the fraction (group 4) increased to 10^9 CFU g^{-1} after day 5 postinfection (Fig. 2). At this time, mice in group 4 were showing signs of dehydration and physical deterioration and were reevaluated every 8 h (Fig. 3). Three mice in group 4 died within the evaluation period, and the rest showed a significant reduction in body temperature (<34°C). At day 7 postinfection, the end point of group 4 was reached and the remaining mice were euthanized (Table 5). The condition of the mice in groups 2 and 3 remained acceptable at 10 days postinfection. Bioluminescent signals from mice in groups 2, 3, and 4 were taken and analyzed in order to compare their light intensities at the specified times. On day 3 of the experiment, all mice were orally infected with 10^8 CFU of EHEC O157. Bioluminescence was monitored on days 3, 5, and 7 after infection. On day 3 after infection, strong bioluminescence was observed in the GI tracts of all of the mice in groups 4 (Fig. 4A) and 2 (Fig. 4F) and two mice in group 3 (Fig. 4C). There was no significant difference in the bioluminescence values (relative light unit counts per square centimeter per second) of any of the groups of mice at day 3 postinfection, as shown in

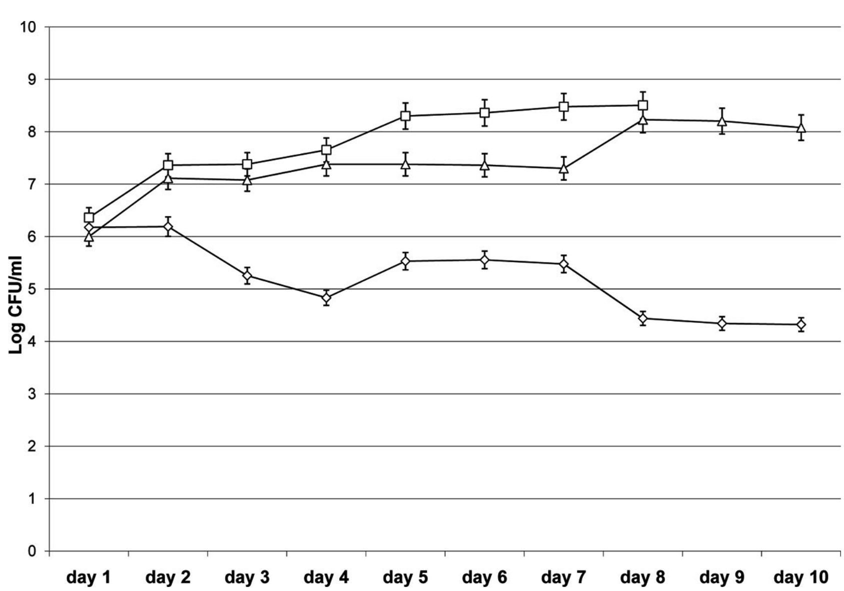

FIG. 2. Average daily fecal shedding of EHEC C4 by group 2 (probiotic-EHEC) (◇), group 3 (EHEC-probiotic) (△), and group 4 (positive control) (□) mice. The data are the mean (± standard deviation) daily fecal shedding values of the groups (n = 5).

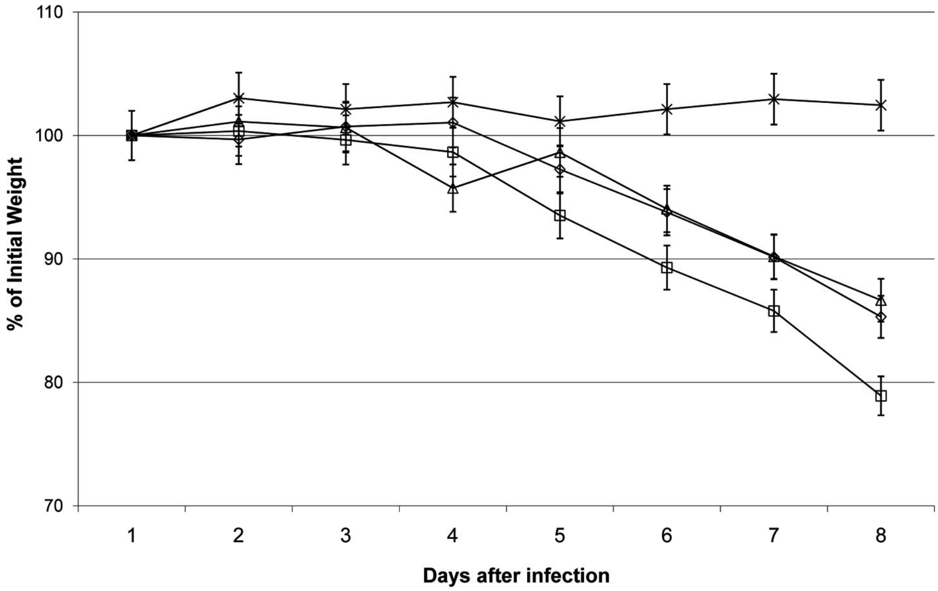

FIG. 3. Body weights of mice during the week following a challenge, indicated as percentages of their initial weights. Values for group 1 (negative control) (×), group 2 (probiotic-EHEC) (◇), group 3 (EHEC-probiotic) (△), group 4 (positive control) (□) mice are shown. The data are the mean (± standard deviation) daily weights of the group ($n = 5$).

Table 4. However, significant differences were observed after day 5 postinfection, as one mouse in group 2 (Fig. 4G) and two mice in group 3 (Fig. 4D) showed no bioluminescent signal. However, the mouse producing the positive signal in group 3 (Fig. 4D) exhibited strong bioluminescence compared to the weak bioluminescent signal emanating from the two mice in group 2 (Fig. 4G). Parallel analyses of the calculated bioluminescence values of all five mice in each group were done, and we observed statistically significantly different values ($P < 0.05$) after day 5 postinfection, when group 4 showed 21,965 ± 4,871 cts $(cm^2\ s^{-1})^{-1}$, compared with the 2,176 ± 635 and 792 ± 82 cts $(cm^2\ s^{-1})^{-1}$ displayed by groups 3 and 2, respectively (Table 4). The bioluminescence observed at day 7 postinfection was greatly decreased in both probiotic-treated groups (Table 4), indicating that the probiotic La-5 fraction is capable of inhibiting EHEC O157 attachment to intestinal epithelial cells (Fig. 4E and H). It has been proposed that the presence of probiotic bacteria in the host GI tract enhances immunity, thereby protecting the host against bacterial infections (11, 13, 31, 32). Taking into account the strain specificity of probiotics (2), we selected and employed CFSM from a probiotic bacterium that downregulated virulence-related genes of EHEC in vitro (29). Because of the already established ability of probiotic cells to protect animal and human hosts once present in their GI tracts (14–16, 30, 33, 38), our work focused on the role of probiotic-secreted molecules in the control of infection. These molecules may employ alternative molecular mechanisms in order to confer enhancement of the immune system, protection against bacterial infection, and/or quorum quenching strategies. It is reasonable to expect that microorganisms employ an array of strategies in order to survive in a complex environment.

TABLE 5. Mouse average body condition scores and survival rates 7 days after a challenge with EHEC C4

Exptl group	Body temp (°C)[a]	Rough coat[b]	Lethargy[b]	No. of survivors/ total by day 5[b]
1, negative control	38.2 ± 0.17	–[c]	–	5/5
2, probiotic-EHEC	33.3 ± 1.7	+	–	5/5
3, EHEC-probiotic	33.6 ± 1.3	++	–	5/5
4, positive control	30.9 ± 1.3	+++	+++	2/5

[a] Data are means ± standard deviations of three group ($n = 5$) replicates.
[b] Signs of deterioration and survival rates are averages of three group ($n = 5$) replicates.
[c] A plus sign(s) represents the presence and severity of signs of deterioration, and a minus sign represents the absence of a sign of deterioration.

Conclusion. The results of our tissue culture assays showed an important reduction in the number of cells able to attach. In addition, the results of our EHEC O157 mouse model experiment revealed that the probiotic *L. acidophilus* La-5 produces molecules that remain active against EHEC attachment to the GI epithelial cells of ICR mice. Complementary in vivo studies could aid in the determination of the extent of the capacity of CFSM to protect against GI tract infections, once the precise nature of the active molecules is determined. After basic analysis of the CFSM, we found that the active fractions consist of heat-stable small molecules that may well be of a proteinaceous nature. Unfortunately, our knowledge of the biochemical and genetic mechanisms responsible for the production of La-5 biologically active molecules against EHEC remains disappointingly scant. The next step will be directed toward the analysis of possible molecule candidates secreted by La-5 and other probiotic bacteria.

Animal hosts are frequently exposed to microbial pathogens, and infection seems to vary from individual to individual. This occurrence stresses that any intrinsic or extrinsic factor could modify the infection outcome by changing the balance between

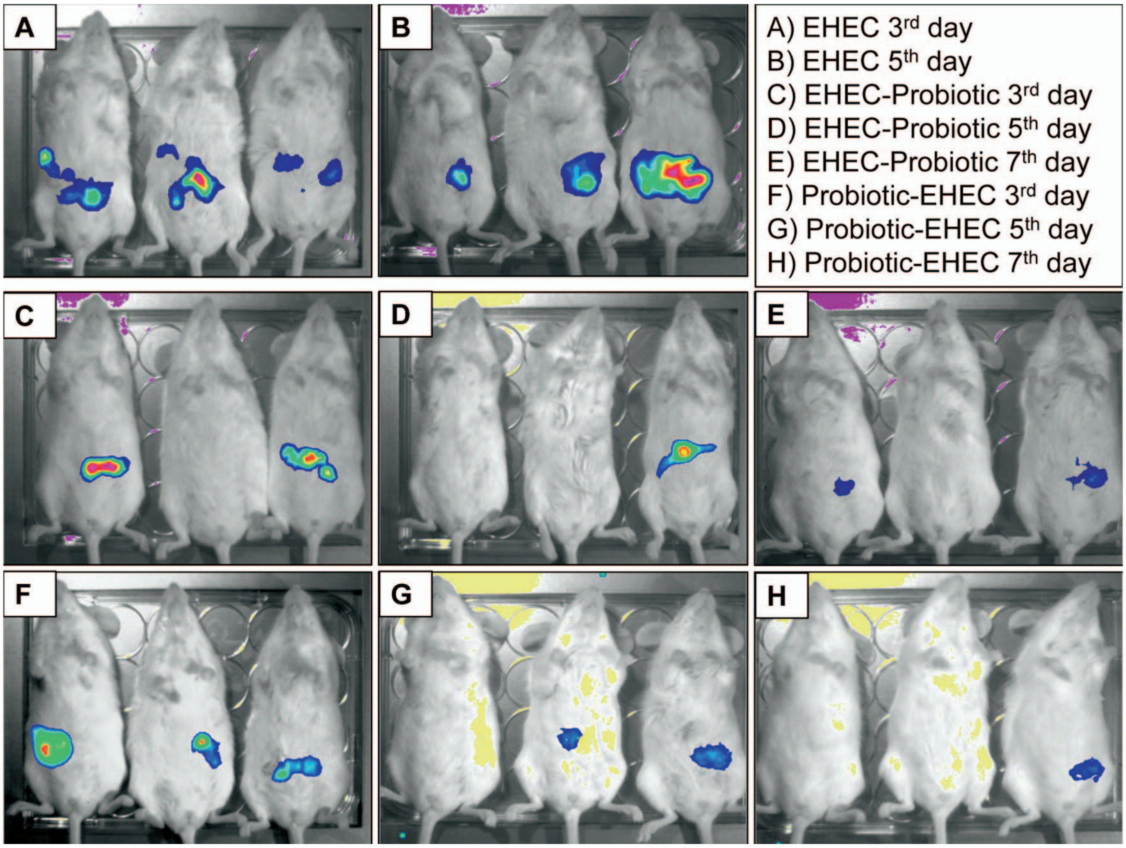

FIG. 4. Bioluminescence images of mice infected with 10^8 CFU of EHEC C4. Images were obtained on days 3, 5, and 7 postinfection. Areas in which luminescent EHEC O157 is present are shown as color overlay.

the host defense system and the virulence capacity of the pathogen. We have shown that the probiotic *L. acidophilus* La-5 is capable of modifying EHEC virulence in vitro and in vivo and that it secretes a molecule(s) able to alter its QS signaling system. Microorganisms have acquired signal interference mechanisms in order to modify their environment to provide a more competitive niche (25). Natural signal interference could be a possible mechanism by which probiotics keep pathogens in check, and their use against human and animal pathogens could provide excellent therapeutic alternatives to antibiotic treatments.

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